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(57) Abstract

Compounds and methods are described for treating cancer, and in particular, compositions and methods for transfecting tumor cells under conditions such that tumor growth is suppressed. Transfecting human *wnt–5a* into tumor cells results in loss of tumorigenicity, permitting a cancer treatment that does not require radiation or toxic chemotherapeutic agents.

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COMPOUNDS AND METHODS FOR TREATING CANCER

This application for patent under 35 U.S.C. 111(a) claims priority to Provisional Application Serial No. 60/031,509, filed November 27, 1996 under 35 U.S.C. 111(b). This invention was made with government support under grant DAMD 17-94-J-4150. The government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to compounds and methods for treating cancer, and in particular, compositions and methods for transfecting tumor cells under conditions such that tumor growth is suppressed.

BACKGROUND

Therapy for cancer has largely involved the use of radiation, surgery and chemotherapeutic agents. However, results with these measures, while beneficial in some tumors, has had only marginal or no effect in many others. Furthermore, these approaches have often unacceptable toxicity.

Both radiation and surgery suffer from the same theoretical drawback. It has been recognized that, given that a single clonogenic malignant cell can give rise to sufficient progeny to kill the host, the entire population of neoplastic cells must be eradicated. See generally, Goodman and Gilman The Pharmacological Basis of Therapeutics (Pergamon Press, 8th Edition) (pp. 1202-1204). This concept of "total cell kill" implies that total excision of a tumor is necessary for a surgical approach, and complete destruction of all cancer cells is needed in a radiation approach, if one is to achieve a cure. In practice this is rarely possible; indeed, where there are metastases, it is impossible.

The term "chemotherapy" simply means the treatment of disease with chemical substances. The father of chemotherapy, Paul Ehrlich, imagined the perfect chemotherapeutic as a "magic bullet;" such a compound would kill invading organisms without harming the host. This target specificity is sought in all types of chemotherapeutics, including anticancer agents.

However, specificity has been the major problem with anticancer agents. In the case of anticancer agents, the drug needs to distinguish between host cells that are cancerous and host cells that are not cancerous. The vast bulk of anticancer drugs are indiscriminate at this level. Typically anticancer agents have negative hematological effects (e.g., cessation of mitosis and disintegration of formed elements in marrow and lymphoid tissues), and immunosuppressive action (e.g., depressed cell counts), as well as a severe impact on epithelial tissues (e.g., intestinal mucosa), reproductive tissues (e.g., impairment of spermatogenesis), and the nervous system. P. Calabresi and B.A. Chabner, In: Goodman and Gilman The Pharmacological Basis of Therapeutics (Pergamon Press, 8th Edition) (pp. 1209-1216).

Success with chemotherapeutics as anticancer agents has also been hampered by the phenomenon of multiple drug resistance, resistance to a wide range of structurally unrelated cytotoxic anticancer compounds. J.H. Gerlach *et al.*, *Cancer Surveys*, 5:25-46 (1986). The underlying cause of progressive drug resistance may be due to a small population of drug-resistant cells within the tumor (e.g., mutant cells) at the time of diagnosis. J.H. Goldie and Andrew J. Coldman, *Cancer Research*, 44:3643-3653 (1984). Treating such a tumor with a single drug first results in a remission, where the tumor shrinks in size as a result of the killing of the predominant drug-sensitive cells. With the drug-sensitive cells gone, the remaining drug-resistant cells continue to multiply and eventually dominate the cell population of the tumor.

Treatment at the outset with a combination of drugs was proposed as a solution, given the small probability that two or more different drug resistances would arise spontaneously in the same cell. V.T. DeVita, Jr., *Cancer*, 51:1209-1220 (1983). However, it is now known that drug resistance is due to a membrane transport protein, "P-glycoprotein," that can confer general drug resistance. M.M. Gottesman and I. Pastan, *Trends in Pharmacological Science*, 9:54-58 (1988). Phenotypically, the tumor cells show, over time, a reduced cellular accumulation of all drugs. In short, combination chemotherapy appears not to be the answer.

What is needed is a specific anticancer approach that is reliable with a wide variety of tumor types. Importantly, the treatment must be effective with minimal host toxicity.

SUMMARY OF THE INVENTION

The invention generally relates to the treatment of cancer, and, more specifically, to the treatment of cancer, including metastases, without radiation or toxic chemotherapeutic agents. In one embodiment, the invention involves compositions and methods for transfecting tumor cells under conditions such that tumor growth is suppressed, and in particular, transfection of nucleic acid comprising the *wnt-5a* gene. Wnt-gene family members are thought to play an important role in cell growth and differentiation. When normal wnt-gene expression is disrupted there is the potential for cell transformation.

The present invention contemplates the use of antisense *wnt-5a* as well as expressing of the *wnt-5a* gene product in tumor cells such that the tumorogenic phenotype is transformed into a non-tumorogenic phenotype. In one embodiment, the present invention contemplates a method, comprising: a) providing i) tumor cells and ii) nucleic acid encoding at least a portion of the *wnt-5a* gene, and b) introducing said nucleic acid into said tumor cells. In one embodiment, said nucleic acid encodes the entire *wnt-5a* gene and said introducing comprises transfection under conditions such that a functional *wnt-5a* gene product is expressed by said tumor cells. Thus, the present invention contemplates a composition comprising tumor cells containing a vector comprising nucleic acid which encodes at least a portion of the *wnt-5a* gene.

DESCRIPTION OF THE DRAWINGS

Figure 1A shows Northern analysis of two different clones of tumor cells stably expressing *wnt-5a*.

Figure 1B is a graph showing growth curves of the stably expressing cells.

Figure 2 is a graph showing tumor volume measurements for transfected tumor cells (and controls) *in vivo*.

Figure 3 shows the telomerase activity of transfected tumor cells (and controls) as measured by the TRAP assay.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

The term "gene" refers to a DNA sequence that comprises control and coding sequences necessary for the production of a polypeptide or precursor thereof. The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired activity is retained. The term "gene" encompasses both cDNA and genomic forms of a given gene. In some embodiments (such as antisense), portions of the gene are contemplated that, while too small to encode a functional protein, are of sufficient size to hybridize to complementary nucleic acid.

The term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene or gene product which displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

The term "oligonucleotide" as used herein is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, usually more than three (3), and typically more than ten (10) and up to one hundred (100) or more (although preferably between twenty and thirty). The exact size will depend on many factors, which in turn depends on the ultimate function or use of the oligonucleotide. The oligonucleotide may be generated in any manner, including chemical synthesis, DNA replication, reverse transcription, or a combination thereof. In one embodiment, the present invention contemplates an oligonucleotide capable of hybridizing to a portion of the *wnt-5a* gene for use in antisense therapy.

As used herein, the term "regulatory element" refers to a genetic element which controls some aspect of the expression of nucleic acid sequences. For example, a promoter is a regulatory element which facilitates the initiation of transcription of an

operably linked coding region. Other regulatory elements are splicing signals, polyadenylation signals, termination signals, *etc.*

Transcriptional control signals in eucaryotes comprise "promoter" and "enhancer" elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription [Maniatis, T. *et al.*, Science 236:1237 (1987)]. Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect and mammalian cells and viruses (analogous control elements, *i.e.*, promoters, are also found in procaryotes). The selection of a particular promoter and enhancer depends on what cell type is to be used to express the protein of interest. Some eukaryotic promoters and enhancers have a broad host range while others are functional in a limited subset of cell types [for review see Voss, S.D. *et al.*, Trends Biochem. Sci., 11:287 (1986) and Maniatis, T. *et al.*, *supra* (1987)].

The terms "expression vector" and "recombinant DNA vector" as used herein refer to DNA sequences containing a desired coding sequence and appropriate DNA sequences necessary for the expression of the operably linked coding sequence in a particular host organism (e.g. mammal). DNA sequences necessary for expression in procaryotes include a promoter, optionally an operator sequence, a ribosome binding site and possibly other sequences. Eukaryotic cells are known to utilize promoters, polyadenlyation signals and enhancers.

The terms "in operable combination", "in operable order" and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

The term "genetic cassette" as used herein refers to a fragment or segment of DNA containing a particular grouping of genetic elements. The cassette can be removed and inserted into a vector or plasmid as a single unit. A plasmid backbone refers to a piece of DNA containing at least plasmid origin of replication and a selectable marker gene (e.g., an antibiotic resistance gene) which allows for selection of bacterial hosts containing

the plasmid; the plasmid backbone may also include a polylinker region to facilitate the insertion of genetic elements within the plasmid.

Because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage, an end of an oligonucleotide is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends.

The term "primer" refers to an oligonucleotide which is capable of acting as a point of initiation of synthesis when placed under conditions in which primer extension is initiated. An oligonucleotide "primer" may occur naturally, as in a purified restriction digest or may be produced synthetically.

A primer is selected to be "substantially" complementary to a strand of specific sequence of the template. A primer must be sufficiently complementary to hybridize with a template strand for primer elongation to occur. A primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being substantially complementary to the strand. Non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the template to hybridize and thereby form a template primer complex for synthesis of the extension product of the primer.

"Hybridization" methods involve the annealing of a complementary sequence to the target nucleic acid (the sequence to be detected). The ability of two polymers of nucleic acid containing complementary sequences to find each other and anneal through base pairing interaction is a well-recognized phenomenon. The initial observations of the "hybridization" process by Marmur and Lane, *Proc. Natl. Acad. Sci. USA* 46:453 (1960) and Doty *et al.*, *Proc. Natl. Acad. Sci. USA* 46:461 (1960) have been followed by the refinement of this process into an essential tool of modern biology.

The complement of a nucleic acid sequence as used herein refers to an oligonucleotide which, when aligned with the nucleic acid sequence such that the 5' end of one sequence is paired with the 3' end of the other, is in "antiparallel association." Certain bases not commonly found in natural nucleic acids may be included in the nucleic acids of the present invention and include, for example, inosine and 7-deazaguanine. Complementarity need not be perfect; stable duplexes may contain mismatched base pairs or unmatched bases. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length of the oligonucleotide, base composition and sequence of the oligonucleotide, ionic strength and incidence of mismatched base pairs.

Stability of a nucleic acid duplex is measured by the melting temperature, or "T_m." The T_m of a particular nucleic acid duplex under specified conditions is the temperature at which on average half of the base pairs have disassociated. The equation for calculating the T_m of nucleic acids is well known in the art.

The term "probe" as used herein refers to a labeled oligonucleotide which forms a duplex structure with a sequence in another nucleic acid, due to complementarity of at least one sequence in the probe with a sequence in the other nucleic acid.

The term "label" as used herein refers to any atom or molecule which can be used to provide a detectable (preferably quantifiable) signal, and which can be attached to a nucleic acid or protein. Labels may provide signals detectable by fluorescence, radioactivity, colorimetry, gravimetry, X-ray diffraction or absorption, magnetism, enzymatic activity, and the like.

The terms "nucleic acid substrate" and nucleic acid template" are used herein interchangeably and refer to a nucleic acid molecule which may comprise single- or double-stranded DNA or RNA.

The term "transfection" as used herein refers to the introduction of DNA (and more typically, foreign DNA) into eukaryotic cells. The present invention specifically contemplates introducing nucleic acid into tumor cells, and more particularly, transfecting tumor cells with a gene of interest.

The term "stable transfection" or "stably transfected" refers to the introduction and

integration of foreign DNA into the genome of the transfected cell. The term "stable transfectant" refers to a cell which has stably integrated foreign DNA into the genomic DNA.

As used herein, the term "gene of interest" refers to a gene inserted into a vector or plasmid whose expression is desired in a host cell. The present invention contemplates that the *wnt-5a* gene is a genes of interest include having therapeutic value.

As used herein, the terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

DESCRIPTION OF THE INVENTION

The present invention relates to compounds and methods for treating cancer, and in particular, compositions and methods for transfecting tumor cells under conditions such that tumor growth is suppressed.

A. Tumor Suppressor Genes

Genetic alterations of chromosomes containing tumor suppressor genes are thought to be contributing to the multi-stage progression of malignant tumors. B. Ponder, "Gene losses in human tumors," *Nature* 335: 400-402 (1988). These alterations may include nonrandom chromosomal deletions or loss of heterozygosity (LOH). Ehlen and Dubeau, "Loss of heterozygosity on chromosomal segments 3p, 6q, and 11p in human ovarian cancers," *Oncogene* 5: 219-223 (1990). The short arm of chromosome 3 (3p) has a particularly high frequency of deletion or rearrangement in human cancers including small cell lung carcinoma, oral squamous cell carcinoma, cervical carcinoma, breast carcinoma, renal cell carcinoma, and uroepithelial cell carcinoma. The loss of heterozygosity determined by restriction fragment length polymorphism (RFLP) analysis occurs consistently in renal cell carcinoma suggesting the loss of one or more tumor suppressor genes which likely play a significant role in renal cell carcinogenesis. Although the

precise location of the 3p tumor suppressor gene(s) is not known, cytogenetic analysis suggests that the region 3p1 1-3p25 likely carries one or more suppressor genes. More specifically, Yamakawa *et al.* mapped one suppressor gene to 3p 13-p 14.2 and another distal to 3p2 1.3 in renal cell carcinoma. Yamakawa *et al.*, "A detailed mapping of the short arm of chromosome 3 in sporadic renal cell carcinoma," *Cancer Res.* 51: 4707-4711 (1991). Another region encompassing 3p12-p14 has been found to dramatically alter tumor growth in nude mice when a fragment containing this region was introduced into a highly malignant nonpapillary renal cell carcinoma cell line. *See* Sanchez *et al.*, "A tumor suppressor locus within 3p14-p12 mediates rapid cell death of renal cell carcinoma *in vivo*," *Proc. Nat. Acad. Sci. USA* 91: 3383-3387 (1994). In bladder cancers, a specific correlation between the loss of chromosome 3p and the development of high grade malignancy has recently been found. Presti *et al.*, "Molecular genetic alterations in superficial and locally advanced bladder cancer, " *Cancer Res.* 51:5404-5409 (1991).

B. Wnt-genes

Human *wnt-5a* has been cloned and mapped to human chromosome 3p14-p21. *Wnt* genes consist of a family of locally acting growth factor-like molecules which are involved in pattern formation, morphogenesis, and cell growth and differentiation. *Wnt1*, *wnt-2* and *wnt-3* are known to be activated by mouse mammary tumor virus (MMTV) proviral insertional mutagenesis in certain mouse mammary tumors. It is not known how some *wnt*-gene family members are involved in cell transformation and tumorigenesis, but it likely involves disruption of normal spatio-temporal *wnt* gene expression. In any event, loss of normal gene expression of *wnt-5a* in the presence of ectopically expressed *wnt-1*, *wnt-2*, or *neu* T appears to correlate with cell transformation. Olson and Papkoff, "Regulated expression of *wnt* family members during proliferation of C57MG mammary cells," *Cell Growth and Differentiation* 5:197-206 (1994).

C. **Transfection of Tumor Cells**

In one embodiment, the invention involves compositions and methods for transfecting tumor cells under conditions such that tumor growth is suppressed, and in particular, transfection of nucleic acid comprising the *wnt-5a* gene. It is not intended that the present invention be limited by the method of transfection. The present invention contemplates that transfection may be accomplished by a variety of means known to the art including (but not limited to) calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics. Moreover, the present invention contemplates transfection *in vitro* and *in vivo*.

It is also not intended that the present invention be limited to a particular type of tumor cell. In one embodiment, the present invention contemplates tumors having deletions for chromosome 3p. A variety of tumor types are available for *in vitro* transfection, including but not limited to breast cancer cells, prostate cancer cells, and renal cancer cells. While the cancer cells of animals in general may be transfected, the present invention specifically contemplates the transfection of human cancer cells. In one embodiment, the present invention contemplates an SV40-immortalized human uroepithelial cell (SV-HUC- I) cell line which showed nonrandom losses of chromosome 3p in association with tumorigenic transformation to high grade cancers. *See* Klingelhutz et al., "Allelic 3p deletions in high-grade carcinomas after transformation in vitro of human uroepithelial cells," *Genes, Chrom. and Cancer* 3: 346-357 (1991)

Transfection (whether stable or transient) can be achieved by use of vectors containing the *wnt-5a* gene, or functional variants thereof. The nucleic acid sequence of *wnt-5a* cDNA and the amino acid sequence of the *wnt-5a* gene product are known (GENBANK Accession No. L20861). Functional variants are those genes containing variations in sequence (*i.e.* non-wild type or mutant sequences) that do not change the function of the nucleic acid (such as in antisense, discussed below) or the function of the resulting *wnt-5a* gene product (*i.e.* the tumor suppressing nature of the gene product). Such variants can be readily tested for functionality using the *in vitro* methods of the

present invention described below (*see* Experimental).

It is not intended that the present invention be limited by the type of vector. A variety of vectors can be used, including but not limited to RSV expression vectors (commercially available from Invitrogen, San Diego, CA). A variety of transfection schemes with RSV expression vectors are known in the art. *See e.g.* U.S. Patent No. 5, 654,140, hereby incorporated by reference.

D. *In Vivo* Targeting

As noted above, the present invention contemplates transfection *in vitro* and *in vivo*. For *in vivo* targeting of tumor cells, a variety of strategies are contemplated including but not limited to systemic exposure to a vector comprising nucleic acid encoding at least a portion of the *wnt-5a* gene.

A number of approaches to delivery are known in the art, including liposomes and ligand targeting. For example, Wu *et al.*, U.S. Patent 5,166,320 (hereby incorporated by reference), discloses tissue-specific delivery of DNA using a conjugate of a polynucleic acid binding agent (such as polylysine, polyarginine, polyornithine, histone, avidin, or protamine) and a tissue receptor-specific protein ligand. For targeting liver cells, Wu suggests asialoglycoprotein (galactose-terminal) ligands. The present invention contemplates targeting said ligand for liver tumor cells, using the above-described expression vectors.

In another example, Wagner *et al.*, *Proc. Natl. Acad. Sci.*, 88:4255-4259 (1991) and U.S. Patent No. 5,354,844 (hereby incorporated by reference) disclose complexing a transferrin-polylysine conjugate with DNA for delivering DNA to cells via receptor mediated endocytosis. Wagner, *et al.*, teach that it is important that there be sufficient polycation in the mixture to ensure compaction of plasmid DNA into toroidal structures of 80-100 nm diameter, which, they speculate, facilitate the endocytic event.

E. Antisense

The present invention contemplates oligonucleotides serving as antisense strands to the *wnt-5a* gene. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. The designation (-) (*i.e.*, "negative") is sometimes used in reference to the antisense strand, with the designation (+) sometimes used in reference to the sense (*i.e.* "positive") strand.

In one embodiment, the oligonucleotides of the present invention are RNA sequences which are complementary to a specific wnt-5a RNA sequence (*e.g.*, wnt-5a mRNA). Antisense RNA may be produced by any method, including synthesis by splicing the gene(s) of interest in a reverse orientation to a viral promoter which permits the synthesis of a coding strand. Once introduced, this transcribed strand combines with the natural mRNA produced by the cell to form duplexes. These duplexes then block either the further transcription of the mRNA or its translation.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); μ (micron); M (Molar); μM (micromolar); mM (millimolar); N (Normal); mol (moles); mmol (millimoles); μmol (micromoles); nmol (nanomoles); g (grams); mg (milligrams); μg (micrograms); ng (nanograms); L (liters); ml (milliliters); μl (microliters); cm (centimeters); mm (millimeters); μm (micrometers); nM (nanomolar);°C (degrees Centigrade); PBS (phosphate buffered saline); U (units); d(days).

EXAMPLE 1

In this example, tumor cells were transfected with a vector comprising full-length human *wnt*-5a cDNA. The cDNA was subcloned into pRSV (Dr. Jackie Papkoff, Sugen, Redwood City, CA) and orientation determined by restriction analysis. SV-HUC-1 and

MC-T16 uroepithelial carcinoma cells were cultured in F12 media supplemented with 1% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin. The media was also supplemented with essential amino acids, ferritin, dexamethasone, insulin, and glutamine as previously described. Cells were grown to 80% confluence before each passage. For gene transfection, passage 20 cells were grown to 50% confluence and the media exchanged for low serum media (Optimem, Gibco). Using 90 μl liposomes (Lipofectin, Gibco) mixed with 10 μg of pRSV*wnt*-5a an/or pSV2neo in a total volume of 150 μl, the cells were transfected overnight at 37^{0}C in 5% $C0_2$. The media was replaced and the cells grown overnight in media with 1% FBS without the addition of Geneticin (G418, Gibco). The cells were then selected in media supplemented with 800 ug/ml G-4I8. Individual clones were isolated, resistant colonies expanded into cell lines, and maintained in media supplemented with 250 μg/mi G418 for eventual RNA extraction to determine gene expression of *wnt*-5a.

Total cellular RNA was isolated from dishes of confluent cells according to the methods of Chomczynski and Sacchi, "Single-step method of RNA isolation by acid guanidinium thiocyante-phenol-chloroform extraction," *Anal. Biochem.* 162:156-159 (1987). Twenty μg of RNA was analyzed on a 1.0 % agarose formaldehyde gel followed by transfer to Hybond-N (Amersham) membrane. The membranes were UV cross-linked (Stratagene) and prehybndized at 42^{0}C for 3-6 hours, and then hybridized at 42^{0}C overnight with full length cDNA *wnt*-5a probes labeled by random priming with [^{32}P]dCTP using 1-2 x 10^6 cpm/ml. The prehybridization and hybridization solutions consisted of 50% formamide, 4x SSPE, 0.2 mg/ml sheared and boiled salmon sperm DNA, 2.5x Denhardt's, and 1% sodium dodecyl sulfate (SDS). Membranes were washed at room temperature twice in 2x SSC, 1% SDS, followed by several washes in 0.1x SSC, 0.1% SDS at 55^{0}C. The membranes were then mounted on 8 x 10 film (Kodak) with an intensifying screen and placed at -80^{0}C up to 5 days.

Figure 1A shows the results of the Northern analysis using a random primed *wnt-5a* cDNA ^{32}P-labeled probe. The results demonstrated expression of the expected 3.2 kb size RNA in several clonally expanded cell lines. Two clones expressing *wnt-5a* shown

in Figure 1A were selected for comparison to the MC-Tl6Ineo transformed and SV-HUC-l/neo nontumorigenic immortal cell lines resistant to G-418.

The growth properties were compared between the MC-T16/neo parental cells and two clones of MC-T16/*wnt*-5a to determine whether *wnt*-5a had any influence on growth kinetics. To determine the growth rates, cell saturation density, population doubling time, and morphologic phenotype of MC-T16/neo cells and *MC*-T16/*wnt*-5a cells, cells were plated in 12-well dishes at a density of 4×10^4 cells per well. The cells were counted every two days for seventeen days, and the growth rate and population doubling time determined from the logarithmic part of the growth curve. The saturation density was determined from the cell number after the cells reached confluence. Morphology was determined by growing cells to confluence and photographed. As shown in Table 1 and Figure 1B, the growth rate of the MC-T16/neo cells expressing *wnt*-5a was similar to MC-T16 cells in the logarithmic phase unlike that for SV-HUC-l/neo cells. That is, the population doubling time of MC-T16/neo cells was 26 hours while that for MC-T16/*wnt*-5a-clone 6 cells was 32 hours and 24 hours for MC-T16/*wnt*-5a-clone 900, in comparison to the doubling time for SV-HUC-1/neo cells which was 56 hours. Cell saturation density at confluence correlated with growth rate. That is, MC-T16/neo parental cells had a saturation density of 8.9×10^5 at confluence, while both MC-T16 clones expressing *wnt*-5a had saturation densities of 9.4×10^5 and 8.0×10^5. This was significantly different than that observed for SV-HUC-1/neo cells which had a saturation density of 3.2×10^5.

Cell lines were plated into 5 cm dishes in standard growth media and grown to confluence with media change every two days (14 days). In triplicate, 4 μCi of ^{3}H-thymidine (methyl ^{3}H, 60-90 Ci/mmol, aqueous, ICN) was added to each dish and incubated for 2 hours at 37^{0}C. The cells were washed twice with PBS followed by addition of 2 ml ice cold 10% trichloroacetic acid (TCA) and incubated for 30 minutes on ice. Cells were then washed with 10% TCA followed by addition of 2 ml of 0.1 N NaOH. The dishes were incubated at 37^{0}C for 30 min and then neutralized with 0.2 ml of lN HCL. The dishes were carefully scraped and the extract added to 10 ml of

Table 1 *Growth characteristics of MC-T16 cells transfected with human wnt-5a.*

Cells at passage 18-20 were seeded at 4 x 10^4/5 cm plastic dishes for thymidine incorporation or 12-well plastic dishes for growth kinetics and saturation density. The saturation density was determined on day 13. To determine growth in 0.35% soft agar, 10^4 cells were suspended and colony formation determined at two weeks. The values shown are mean +/- SE of at least three dishes. The experiments were repeated three times with similar results.

Cell type	Generation time (h)	Saturation density (total cells x 10^5)	^{3}H-thymidine incorporation (cpms)	Colony formation (%)
SV-HUC-1	56	3.2 +/- 0.1	8,212	0
MC-T16	26	8.9 +/- 0.8	15,110	9.7 +/- 0.3
MC-T16 hwnt-5a-6	32	9.4 +/- 0.7	11,420	0
MC-T16 hwnt-5a-900	24	8.0 +/- 0.6	10,435	0.06 +/- 0.03

scintillation fluid for counting. A fourth dish of cells was grown in parallel to determine total protein and the counts normalized to total protein.

The results (Table 1) reflect the summary of three separate experiments. These results suggests that the expression *of wnt-5*a in MC-T16 cells does not alter growth kinetics significantly.

The parental SV-HUC-l/neo cells characteristically retain many of the features associated with normal epithelial cells in culture. The isolated SV-HUC-l/neo clone used for these experiments no longer grew at confluence unlike that for SV-HUC-l/neo pooled clones which become tightly packed at confluence. MC-T16/neo carcinoma cells continue to grow at confluence, are refractive, and have a spindle cell phenotype. However, when two different clones of MC-T16/*wnt-5a* cells were examined, the cells at confluence become less spindle shaped, more flattened, and pleiomorphic, similar to the SV-HUC-l/neo cells. These findings suggest that MC-T16 bladder cancer cells ectopically expressing *wnt*-5a are more differentiated than the MC-T16/neo cells.

EXAMPLE 2

In this example, the transfected cells of Example 1 were examined in an anchorage dependance assay. In triplicate, 10^4 cells were plated in 0.35% agar (Noble) suspension using standard media over a previously poured 0.5% agar base in 12-well dishes. A G-418 selected SV-HUC-1/neo clone which does not grow in soft agar was used as a negative control. G-418 resistant MC-T16/neo cells were used as a positive control. Two clones of MC-T 16/*wnt*-5a were plated for comparison. Colony formation was determined daily for two weeks. Media was added to the wells as needed. Three separate soft agar assays were done as above and the results pooled. MC-TI6-neo cells grow in 0.35% agar with a cloning efficiency of 9.7% compared to no growth in corresponding parental G418 selected SV-HUC-l/neo cells (Table 1). The expression of *wnt*-5a in MC-T16 cells re-establishes anchorage dependent growth under these conditions in two different clones. The experiments were repeated three times with similar results.

EXAMPLE 3

In this example, tumorigenesis of *wnt-5a* transfected tumor cells were examined *in vivo*. The tumorigenic potentials of G-418 selected positive control MC-T16/neo cells, negative control SV-HUC-1/neo cells and two clones of MC-T16/*wnt*-5a were tested by inoculations into 4-6 week old female athymic nude mice (Charles River). Mice were housed in sterile bubbles in a temperature and humidity controlled room. Inoculations of 2×10^6 cells/site in a total volume of 0.1 ml were made s.c. in the right dorsal quadrant. The animals were examined weekly. Tumors were removed at six months or when necrotic or when 1.5-2 cm in diameter. Representative sections of tumors were fixed in formalin for histologic preparation and stained with H and E. Fixed and stained tumors were examined blindly by three pathologists. Representative pieces of some tumors were used to initiate tumor cell lines using an explant technique. Tumor tissue was cut into 1 mm^2 explant fragments after washing in PBS which were then plated onto tissue culture grade dishes in F-12 1% fetal bovine serum supplemented media without G-418. After I week, the explants were grown in the presence of 250 μg/ml G-418 and the cells expanded for later analysis.

Subcutaneous inoculation of MC-T 16/neo uroepithelial cells resulted in tumor formation after 4 weeks in 9/10 animals. Spontaneous tumor regression occurred in 4 mice which has been previously described for tumors derived from this cell line, and one 1 cm x 1 cm tumor was removed for analysis before the end of the experiment. The remaining tumors grew slowly until the third month when tumor growth rapidly accelerated (Figure 2). These tumors were removed when greater than 1.5 cm x 1.5cm or became necrotic. No tumors grew in 10 mice inoculated with SV-HUC-1/neo cells or in 10 mice inoculated with MC-T16/*wnt*-5a-clone 6 cells. However, 5/5 mice inoculated with MC-T16/*wnt*5a-clone 900 cells grew tumor after a lag time of six weeks. Tumor regression occurred in one animal and one 0.6 cm x 0.8 cm necrotic tumor was removed from another animal. At three months, these tumors grew to a maximum size which never enlarged greater than 1 cm x 1 cm over the following three months (Table 2). The tumors that were examined from mice inoculated with MC-T16/neo cells were attached to

Table 2 *Tumorigenicity of human uroepithelial cells in athymic nude*
Cells at passage 18-20 were grown and 2 x 10^6 cells in 0.1 ml were injected s.c into the right dorsal quadrant of 4-6 week old female athymic nude mice.

Cell lines	Tumor formation	Tumor regression	Tumor size > 1 cm x 1cm
SV-HUC-1	0/10	0/0	0/0
MC-T16	9/10	4/9	4/5
MC-T16 hwnt-5a-6	0/10	0/0	0/0
MC-T16 hwnt-5a-900	5/5	1/5	0/4

underlying tissue and were vascular, while the tumors that grew in mice inoculated with MC-T16/*wnt*-5a-clone 900 cells were found to be unattached to surrounding tissues and remarkably avascular. Tumors from both groups examined histologically confirmed the gross findings. Furthermore, the tumors expressing *wnt-5a* were 80-90% centrally necrotic compared to MC-T16/neo cell derived tumors which were 10-20% necrotic even when considering that the latter tumors grew to a much larger size.. At higher magnification, little stroma was apparent in MC-T16/*wnt*-5a derived tumors compared to MC-T16/neo tumors.

Although the present invention is not limited to a precise mechanism by which *wnt*-5a is able to suppress tumorigenicity, it is of interest that the MC-T16/*wnt-5a* cells which did form tumors, formed tumors which were relatively avascular with extensive necrosis compared to the parental MC-T16/neo derived tumors. This raises the possibility that *wnt*-5a signalling involves pathways important for neo-angiogenesis. Interestingly, the tumors formed from MC-T 16/*wnt*-5a cells also were remarkably devoid of stroma. The relationship between stroma, cell adhesion molecules, and angiogenesis is well documented. Tuszynski and Nicosia, "The role of thrombospondin-1 in tumor progression and angiogenesis," *Bioessays* 18: 71-76 (1996). It is possible that *wnt*-5a compromises angiogenesis by altering cell adhesion, which would offer an explanation why the other MC-T16/*wnt*-5a expressing clone did not grow at all in athymic nude mice.

EXAMPLE 4

Reports have shown that the process of transformation/neoplasia is associated with the activation of telomerase, a ribonucleoprotein enzyme complex that adds telomeric repeats (hexanucleotide 5'-TTAGGG-3') to the ends of replicating chromosomes, or telomeres, and that another member of the *Wnt* family has been shown to regulate telomerase. In this example, telomerase activity is compared between two different G-418 resistant clones of parental MC-T16/neo cells and two clones of MC-T16/*wnt*-5a cells by the primer extension telomere repeat amplification protocol (TRAP) assay in which telomerase synthesizes telomeric repeats onto oligonucleotide primers.

Two different MC-T16/neo cell lines were compared for telomerase activity to two different MC-T16/*wnt*-5a cell lines. Subconfluent cultures were used to prepare the detergent 3-[(cholamidopropyl) dimethylaminonio]-1-propanesulfonate (CHAPS) extracts. Telomerase enzyme activity was measured by using a PCR-based telomeric repeat amplification protocol (TRAP) kit from Oncor, Inc. as per manufacturer's instructions. Each reaction product was amplified in the presence of an internal TRAP assay standard (ITAS, 36 bp). The TRAP reaction products were separated by 10% PAGE, dried, and autoradiographed. The basal levels of telomerase activity (ladder formation) was measured by serial dilution of the protein extracts, and an appropriate range of protein concentration selected that produced a linear response. Each set of TRAP assay included control reaction tubes without any extract, extracts treated with RNase A (200 μg/ml), and positive assay control TSR8 template (2 μl/tube). To quantitate the levels of telomerase activity, the average optical density of first six TRAP bands after primer band was used as a ratio to ITAS band.

The results are shown in Figure 3. The results indicate no change in telomerase activity in either MC-T16/*wnt*-5a expressing cell lines.

Telomerase activity is believed to correlate with cell immortalization, cell transformation, and with tumorigenesis. Telomerase is a ribonucleoprotein complex which adds telomeric repeats onto chromosomal ends. This is thought to protect the ends against nucleases and ligases thus maintaining stability, and to counter shortening of telomere length as a result of DNA replication. Most human tumor cells have enhanced telomerase activity when compared to normal somatic cells. It has recently been reported that telomerase is activated in *wnt*-1 mediated mouse mammary tumors. Broccoli et al., "Telomerase activation in mouse mammary tumors: Lack of detectable telomere shortening and evidence for regulation of telomerase RNA with cell proliferation," *Mol. Cell. Biol.* 16: 3765-3772 (1996). This is interesting considering the endogenous expression of *wnt*-5a decreases 2-3-fold in *wnt*-1 transformed C57MG mouse mammary epithelial cells. Since differentiation of tumor cells has been found to correlate with repression of telomerase activity, it was reasonable to attempt to determine the effect of

wnt-5a on telomerase activity. This is particularly true given that *wnt*-5a transfected into nontumorigenic renal cell carcinoma cells (RCC23) causes telomerase activity to be repressed (data not shown). One plausible explanation for the differences between the cells is that the parental cell line (SV-HUC-l), unlike RCC23 cells, has been immortalized by SV4O which has been previously shown to activate telomerase and stabilize telomere shortening. There also is evidence for up-regulation of telomerase RNA which is dependent on cell proliferation. Furthermore, telomerase activity in tumor cells increases during S phase with less activity detected during Go/Gl. It would be of interest to determine whether SV40 alters cell-cycle dependent telomerase activity.

From the above, it should be clear that transfecting human *wnt-5a* into tumor cells results in loss of tumorigenicity *in vivo* and suppresses anchorage independent cell growth in soft agar. This demonstrates that human *wnt-5a* is a novel tumor suppressor gene and is likely to be one of the suppressor genes deleted or rearranged on chromosome 3p.

CLAIMS

1. A method, comprising:

a) providing:

i) tumor cells and

ii) nucleic acid encoding at least a portion of the *wnt-5a* gene, and

b) introducing said nucleic acid into said tumor cells.

2. The method of Claim 1, wherein said nucleic acid encodes the entire *wnt-5a* gene.

3. The method of Claim 2, wherein said introducing comprises transfection under conditions such that a functional *wnt-5a* gene product is expressed by said tumor cells.

4. The method of Claim 3, wherein said conditions comprise transfection wherein said nucleic acid is in an expression vector.

4. The method of Claim 3, wherein said tumor cell is stably transfected.

5. The method of Claim 3, wherein said gene product is transiently expressed.

6. The method of Claim 3, wherein said gene product is stably expressed.

7. The method of Claim 1, wherein said tumor cells have deletions for chromosome 3p.

8. A method, comprising:

a) providing:

i) tumor cells and

ii) an expression vector comprising nucleic acid encoding the full-length *wnt-5a* gene, and

b) introducing said vector into said tumor cells.

9. The method of Claim 8, wherein said introducing comprises transfection under conditions such that a functional *wnt-5a* gene product is expressed by said tumor cells.

10. The method of Claim 9, wherein said tumor cell is stably transfected.

11. The method of Claim 9, wherein said gene product is transiently expressed.

12. The method of Claim 9, wherein said gene product is stably expressed.

13. The method of Claim 8, wherein said tumor cells have deletions for chromosome 3p.

14. A composition, comprising tumor cells containing a vector comprising nucleic acid which encodes at least a portion of the *wnt-5a* gene.

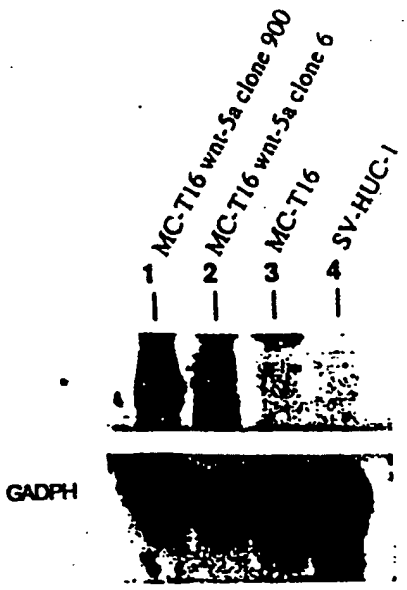
15. The composition of Claim 14, wherein said nucleic acid encodes the entire *wnt-5a* gene.

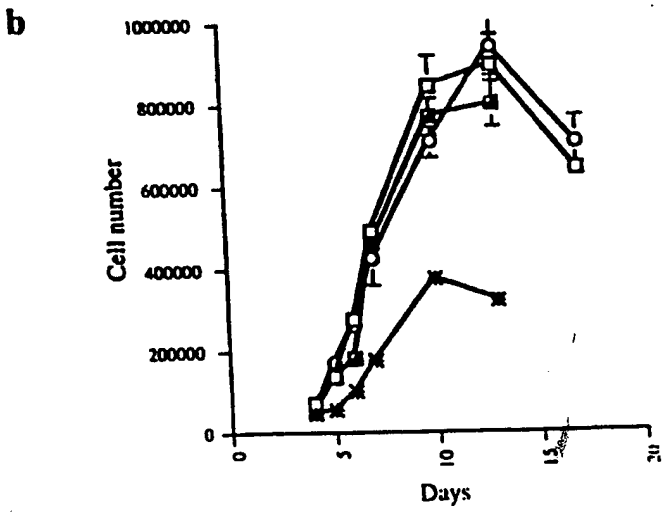
16. The composition of Claim 15, wherein said tumor cells express a functional *wnt-5a* gene product.

17. The composition of Claim 16, wherein said gene product is transiently expressed.

18. The composition of Claim 16, wherein said gene product is stably expressed.

19. The composition of Claim 14, wherein said tumor cells have deletions for chromosome 3p.


1 MC-T16 wnt-5a clone 900
2 MC-T16 wnt-5a clone 6
3 MC-T16
4 SV-HUC-1
GADPH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Cell number
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15
Days

FIGURE 1

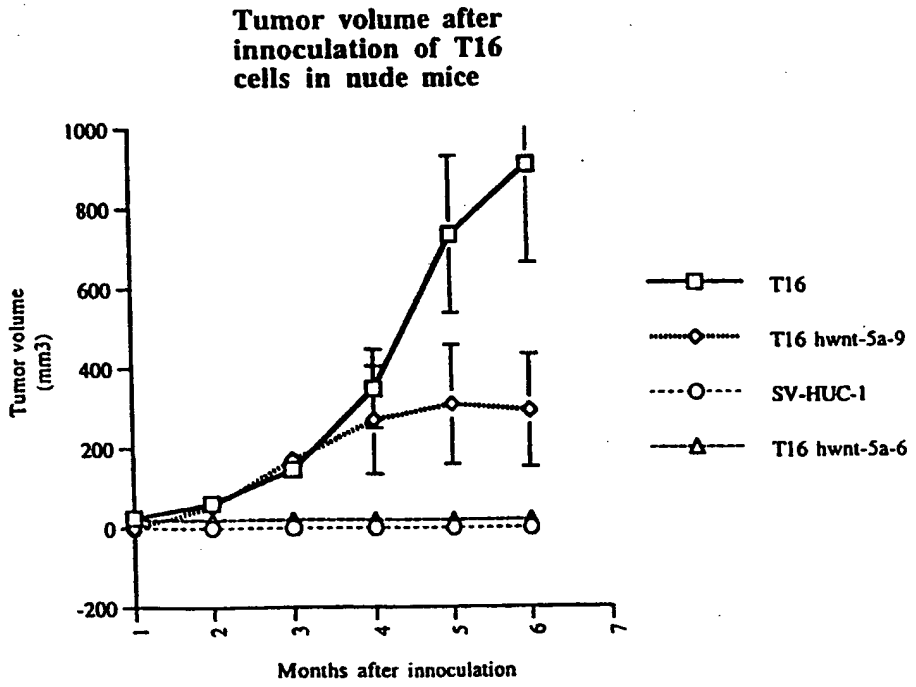
Tumor volume after innoculation of T16 cells in nude mice
Tumor volume (mm3)
1000
800
600
400
200
0
-200
1
2
3
4
5
6
7
Months after innoculation
T16
T16 hwnt-5a-9
SV-HUC-1
T16 hwnt-5a-6

FIGURE 2

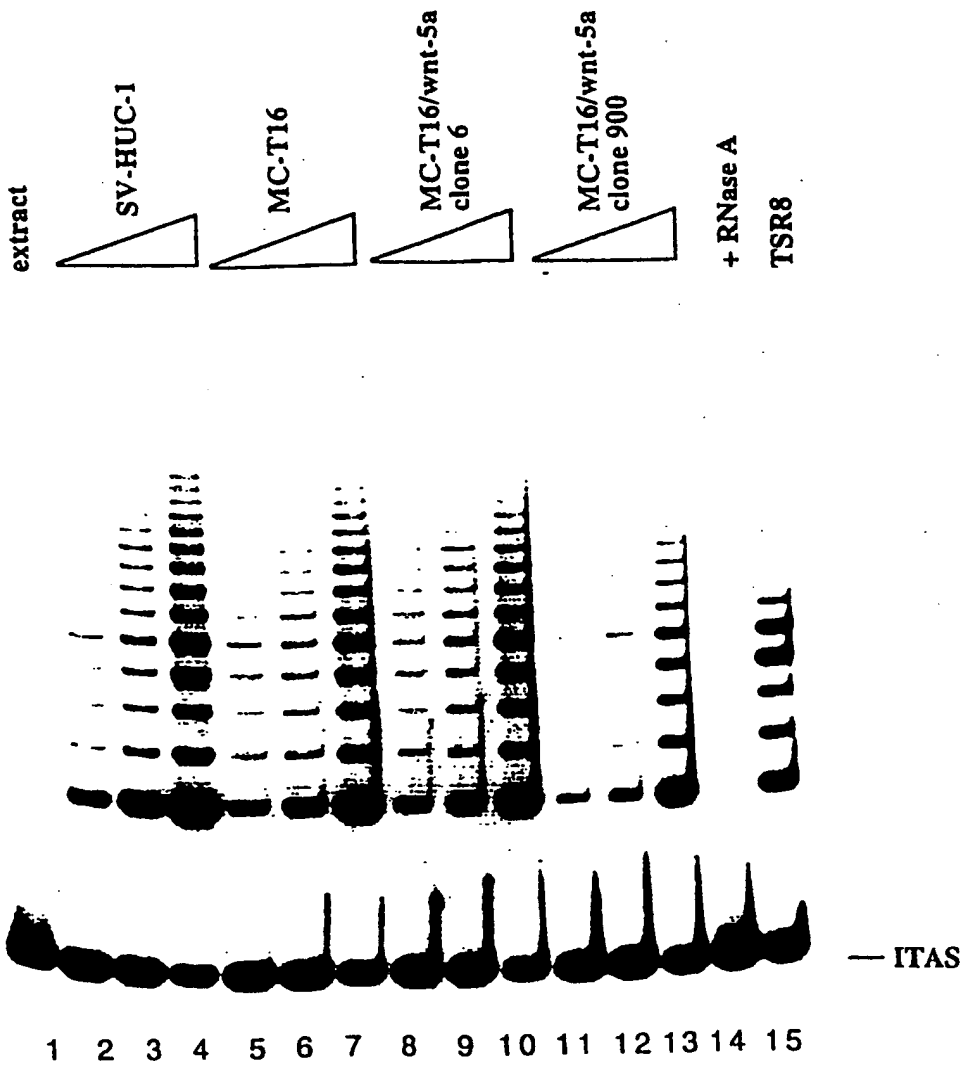
extract
SV-HUC-1
MC-T16
MC-T16/wnt-5a clone 6
MC-T16/wnt-5a clone 900
+ RNase A
TSR8
— ITAS
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

FIGURE 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/22087

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 5/00, 5/02, 5/06, 5/08, 15/00; C12Q 1/68
US CL :435/6, 172.1, 325, 366, 377; 514/44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6, 172.1, 325, 366, 377; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, MEDLINE, BIOSIS, EMBASE, SCISEARCH, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HUGUET, E. et al. Regulation of Wnt5a mRNA Expression in Human Mammary Epithelial Cells by Cell Shape, Confluence, and Hepatocyte Growth Factor. The Journal of Biological Chemistry. 26 May 1995. Vol. 270. No. 21. pages 12851-12856, see entire document.	1-19
Y	IOZZO, R, et al. Aberrant Expression of the Growth Factor Wnt-5a in Human Malignancy. Cancer Research. 15 August 1995. Vol. 55. pages 3495-3499, see entire document.	1-19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
11 FEBRUARY 1998

Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/22087

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	BUI, T. et al. Expression of Wnt5a is Downregulated by Extracellular Matrix and Mutated c-Ha-ras in the Human Mammary Epithelial Cell Line MCF-10A. Biochemical and Biophysical Research Communications. 1997. Vol. 239. pages 911-917. , see entire document.	1-19
Y	VIDER, B. et al. Evidence for the Involvement of the Wnt 2 Gene in Colorectal Cancer. Oncogene. 1996. Vol. 12. pages 153-158, see entire document.	1-19
Y	OLSON, D. et al. Regulated Expressiuon of Wnt Family Members During Proliferation of C57mg Mammary Cells. Cell Growth and Differentiation. February 1994. Vol. 5. pages 197-206, see entire document.	1-19